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ORIGINAL PAPER

L. Domingues · J.A. Teixeira · M. Penttilä · N. Lima

Construction of a flocculent *Saccharomyces cerevisiae* strain secreting high levels of *Aspergillus niger* β-galactosidase

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Abstract A flocculent *Saccharomyces cerevisiae* strain secreting *Aspergillus niger* β-galactosidase activity was constructed by transforming *S. cerevisiae* NCYC869-A3 strain with plasmid pVK1.1 harboring the *A. niger* β-galactosidase gene, *lacA*, under the control of the *ADH1* promoter and terminator. Compared to other recombinant *S. cerevisiae* strains, this recombinant yeast has higher levels of extracellular β-galactosidase activity. In shake-flask cultures, the β-galactosidase activity detected in the supernatant was 20 times higher than that obtained with previously constructed strains (Domingues et al. 2000a). In bioreactor culture, with cheese-whey permeate as substrate, a yield of 878.0 nkat/gsubstrate was obtained. The recombinant strain is an attractive alternative to other fungal β-galactosidase production systems as the enzyme is produced in a rather pure form. Moreover, the use of flocculating yeast cells allows for enzyme production with high productivity in continuous fermentation systems with facilitated downstream processing.

Introduction

β-Galactosidase [β-D-galactoside galactohydrolases, (E.C.3.2.1.23)] has many applications in the food industry. The technological importance of this enzyme is mainly due to the problems associated with whey disposal, lactose crystallization in frozen concentrated desserts, and milk consumption by lactose-intolerant populations. Moreover, the hydrolysis of lactose produces the soluble monosaccharides glucose and galactose and is associated with a considerable increase in sweetness (Shukla 1975; Gekas and López-Leiva 1985).

L. Domingues · J.A. Teixeira · N. Lima (✉)
Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710–057 Braga, Portugal
e-mail: nelson@iec.uminho.pt
Tel.: +351-253-604400, Fax: +351-253-678986

M. Penttilä
VTT Biotechnology, P.O.Box 1500, FIN-02044 VTT, Finland

Certain bacteria, yeasts and moulds produce β-galactosidase. The microbial origin determines the main characteristics of the enzyme, such as temperature and pH optima. Bacterial and yeast β-galactosidases are neutral enzymes while fungal β-galactosidases are acidic. Fungal β-galactosidases are particularly interesting for whey hydrolysis (Nevalainen 1981), not only because of their acidic pH optimum, but also due to their activity at high temperatures (40–65 °C), minimizing the risk of contamination.

Genetically engineered strains of *Saccharomyces cerevisiae* secreting an *Aspergillus niger* β-galactosidase have been constructed (Kumar et al. 1992; Ramakrishnan and Hartley 1993). However, these strains lack biotechnological application as they have low growth rates (Kumar et al. 1992) or, despite good growth and fermentation characteristics, exhibit low plasmid stability (10%, Ramakrishnan and Hartley 1993). Recently, we reported the construction of flocculent and non-flocculent brewer's yeasts secreting an *A. niger* β-galactosidase (Domingues et al. 2000a). The feasibility of producing extracellular β-galactosidase with flocculent cells was shown, generating new perspectives for production of this enzyme in continuous high-cell-density systems with high productivity (Domingues et al. 2000b).

In this work, a flocculent yeast strain secreting levels of *A. niger* β-galactosidase higher than those previously reported for recombinant *S. cerevisiae* cells (Kumar et al. 1992; Ramakrishnan and Hartley 1993; Domingues et al. 2000a) was constructed. The β-galactosidase activity produced is comparable to that from fungi but with higher purity, thereby minimizing the need for purification steps. Moreover, as the producing cells are flocculent, a continuous high-cell-density system can be taken advantage of to produce heterologous β-galactosidase (Domingues et al. 2000b).

Materials and methods

Strains and plasmids

The cloning vector used for yeast transformation was the previously described pVK1.1 (Kumar et al. 1992). This plasmid is based on the YEp24 plasmid and the *lacA* gene from *A. niger* expressed under the *ADH* promotor and terminator. *Escherichia coli* DH5α (F⁻*rec*A1*end*A1*thi*-1*gyr*A96*hsd*R17*sup*E44*rel*A1ϕ89d*lac*ZΔM15λ⁻) was used as the recipient strain for plasmid amplification. The flocculent wild-type haploid *S. cerevisiae* strain NCYC869 (*MatαFLO1*) was mutagenized to uracil auxotrophy by ultraviolet radiation (Lima et al. 1995), and the *ura3* mutation confirmed by genetic complementation with the *URA3* gene (Venâncio et al. 1999). The auxotrophic *S. cerevisiae* strain NCYC869-A3 (*MatαFLO1ura3*) was used for transformation experiments.

Media and growth conditions

For cultivating bacterial cells, LB medium (1% casein, 0.5% yeast extract, 0.5% NaCl) supplemented with 100 µg ampicillin/ml was used. Yeasts were grown in complete YEPG medium (2% glucose, 2% peptone, 1% yeast extract) or minimal YNB medium (2% carbon source: glucose, galactose or lactose; 0.67% Difco yeast nitrogen base without amino acids) at 30 °C. The *ura3* auxotrophic mutants were grown in YNB glucose medium supplemented with 50 µg uracil/ml.

Yeast transformation

Yeast was transformed by the lithium-acetate method according to Hinnen et al. (1978) with the modifications referred to in Schiestl and Gietz (1989).

β-Galactosidase activity assays

Screening for β-galactosidase activity in yeast clones

The presence of β-galactosidase activity in the yeast clones was tested by a microplate assay with *p*-nitrophenyl-β-D-galactopyranoside (pNPG, Sigma) as substrate, as previously described (Domingues et al. 1997). One hundred µl of each cell culture were plated in a well and to each well was added 200 µl SDE (0.05 M Tris/HCl, pH 7.5–8.0; 0.01 M EDTA; 1 M KCl and 0.05 M 2-mercaptoethanol), 5 µl of 0.1% (w/v) SDS and 5 µl of chloroform. β-Galactosidase activity was detected by adding 50 µl of 4 mg pNPG/ml per well, incubating the plates at 65 °C for 30 min and detecting positive wells by their yellow color (Domingues et al.2000a).

Enzyme activity measurements

The β-galactosidase activity in the culture medium was measured as the release of *p*-nitrophenol from pNPG, as previously described (Domingues et al. 2000a). Samples were incubated with 1.7 mM substrate in 0.075 M Na-acetate buffer, pH 4.5, for 10 min at 65 °C. The pH was raised to 10 with 1 M Na_2CO_3 and the activity was measured spectrophotometrically at 405 nm on a scanning multiwell spectrophotometer (SLT Spectra; Bailey and Linko 1990). One unit of activity was defined as the amount of enzyme that hydrolyzed 1 nmol pNPG/min at 65 °C. One nkat is defined as the amount of the enzyme required to liberate 1 nmol of pNPG per second.

Batch cultures and culture media

The recombinant yeast was maintained at 4 °C on slants or at –80 °C in permanent culture on YNB selective medium. SSlactose medium of the following composition was used: 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.2% yeast extract; 5% or 10% lactose. Sweet cheese-whey permeate from a Portuguese dairy industry was obtained by ultrafiltration; the lactose concentration was approximately 5%. The cheese-whey permeate was further concentrated by nanofiltration and a product with approximately 10% lactose was obtained and tested. Yeast cultures were incubated at 30 °C by rotary shaker with 150 rpm agitation, in 100 ml of culture medium in 250-ml Erlenmeyer flasks. Alternatively, a 2-l bioreactor (Braun, Biostat M) fitted with agitation and aeration control as well as temperature and pH measurement and control were used. The temperature was maintained at 30 °C and the pH at 4.0 by automatic addition of an ammonia solution. The agitation speed was set at 150 rpm and the bioreactor was aerated with filtered air at a flow rate of 0.5 vvm.

Biomass determination

Biomass concentrations were measured as dry-weight (DW) and/or absorbance methods. The DW was determined by filtering the sample through 0.2-µm filter-paper and then drying at 105 °C for 24 h. The absorbance was measured at 600 nm on a scanning multiwell spectrophotometer (SLT Spectra) and compared to a previously constructed standard curve for absorbance vs DW. Samples were treated with deflocculation solution (NaCl 1.5%, pH 3.0) before the absorbance was read.

Lactose, glucose, galactose and ethanol measurements

Total reducing-sugar concentration was determined by the dinitrosalicilic acid method (Miller 1959). Lactose, glucose, galactose and ethanol concentrations were determined by HPLC (PL Hi-Plex Pb Column). The solvent was ultrapure water, at a flow rate of 0.6 ml/min, while for detection a refractive index detector was used. Temperature was maintained at 80 °C.

Results

Construction and characterization of the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1

The flocculent strain *S. cerevisiae* NCYC869-A3 was transformed with the vector pVK1.1 containing the *A. niger* β-galactosidase-encoding gene under the *ADH1* promoter. The transformants were selected in YNB/galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). In this medium, only the clones that have been transformed with the selectable marker URA3 are able to grow. Furthermore, the presence of β-galactosidase activity can be identified by the blue color of the colonies. All the recombinant colonies obtained were blue. Yeast transformation was further confirmed by plasmid extraction followed by back-transformation into *E. coli*. The flocculation ability of the recombinant strain was identical to that of the host strain.

Comparison with the previously constructed strains

In order to compare the obtained transformants with previously constructed β-galactosidase-producing yeast strains (Domingues et al. 2000a), preliminary batch cultures were set up in shake-flasks containing SSlactose

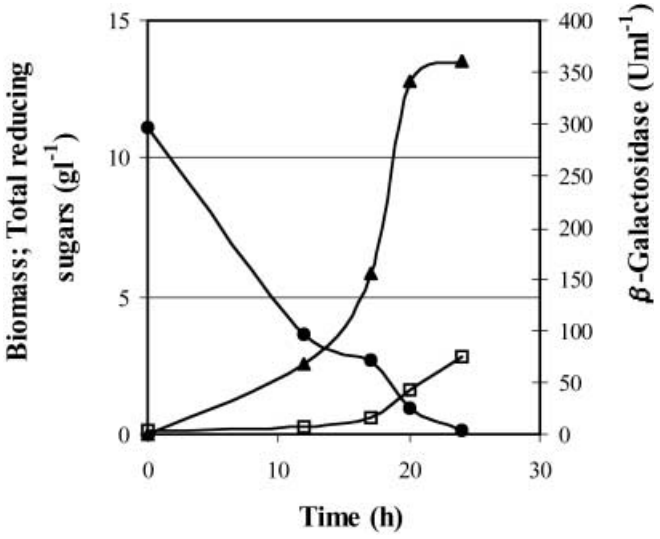

Fig. 1 Typical time course of a batch shake-flask culture using SSlactose (1%) medium and the recombinant *Saccharomyces cerevisiae* NCYC869-A3/pVK1.1. Total reducing sugars (●), biomass concentration (□) and β-galactosidase activity (▲)

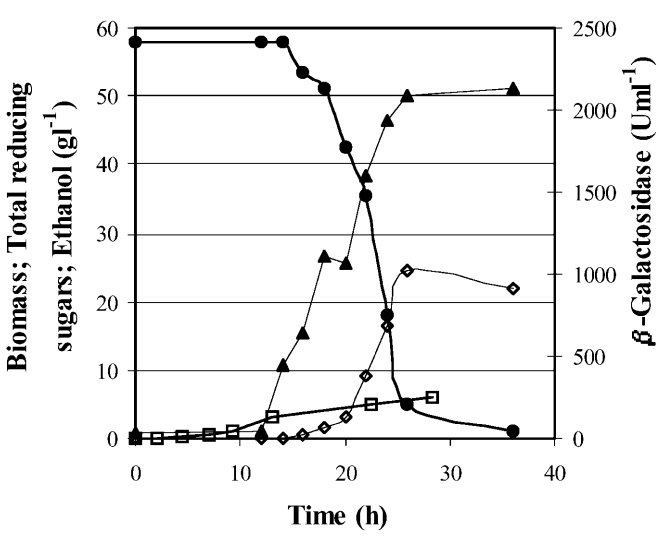

Fig. 2 Typical time course of a batch culture in a 2-l bioreactor using SSlactose (5%) medium with 2 g yeast extract l^{-1} and the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1. Total reducing sugars (●), biomass concentration (□), ethanol concentration (◇) and β-galactosidase activity (▲)

medium with 1% lactose and 0.2% yeast extract. A typical time-course of a batch culture is presented in Fig. 1.

As with the previous constructed strains (Domingues et al. 2000a), the β-galactosidase activity in the culture medium increased with increasing cell concentration, reaching its maximum when cell growth approached the stationary phase. However, a 20-fold increase of β-galactosidase activity was detected in the supernatant.

Bioreactor cultivations on lactose and on cheese-whey permeate

For a better characterization of the recombinant strain, fermentations were done under controlled conditions in a 2-l bioreactor. The semi-synthetic medium SSlactose with 2 g yeast extract l^{-1} and cheese whey permeate were used as substrate. In all the fermentations, stability studies of the plasmid indicated that more than 75% of the population retained the plasmid at the end of the culture period. At this time colonies containing the plasmid were identified by their blue phenotype on 2% lactose/galactose minimal agar plates containing Xgal. This agrees with the pVK1.1 stability reported by Kumar et al. (1992) and is considerably higher than that described for polyploid distiller's yeast also transformed with pVK1.1 (Ramakrishnan and Hartley 1993).

Figure 2 shows a typical time-course for a controlled batch fermentation using an initial lactose concentration of 50 g l^{-1}. The lactose is metabolized in less than 30 h with the production of more than 2,000 U β-galactosidase activity ml^{-1}. Ethanol was also produced at a concentration close to the maximum theoretical yield (25 g l^{-1}). During the first 24 h, the glucose and galactose concentrations detected were residual (Fig. 3), and thus the total reducing-sugar profile presented in Fig. 2 is very similar to that of the lactose concentration in the medium. Glucose and galactose concentrations were found to be always below 2 g l^{-1} (Fig. 3), showing that the monosaccharides enter the cell with no significant accumulation in the culture broth, lactose hydrolysis being the limiting step.

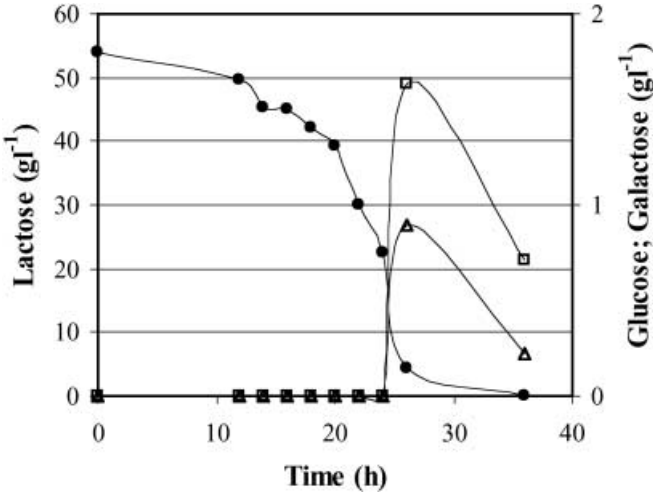

Fig. 3 Sugar profile of the batch culture in 2-l bioreactor using SSlactose (5%) medium with 2 g yeast extract l^{-1} and the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1. Lactose (●), galactose (□), glucose (△) in the medium

Discussion

In this study a flocculent *S. cerevisiae* strain secreting high levels of *A. niger* β-galactosidase was constructed. In shake-flask culture, the β-galactosidase activity de-

Table 1 Comparison of β-galactosidase secreted from recombinant flocculent *Saccharomyces cerevisiae* NCYC869-A3/pVK1.1 cells with enzymes produced by fungi (Macris 1982). *NR* Not reported

Organism	Growth medium	Optimum: pH, T (°C)	Maximum enzyme yield[a]
Aspergillus oryzae[b]	Wheat bran[c]	4.0–5.0, 55–60	150.0
A. oryzae[b]	Starch (4%)[d]	4.0–5.0, 55–60	212.5
Scopulariopsis sp.[b]	Wheat bran[c]	3.6–5.0, 50–65	233.0
Fusarium moniliforme	Wheat bran[c]	3.8–5.0, 50–65	182.0
F. moniliforme	Whey (6%)[e]	3.8–5.0, 50–65	50.0
Alternaria alternata	Whey (6%)[e]	4.5–5.5, 60–70	280.0
Aspergillus niger VTT-D-80144	Wheat bran (4%)[d]	NR, NR	4150.0
S. cerevisiae NCYC869-A3/pVK1.1	Whey permeate (5%)[e]	3.6–5.1, 55–65	878.0
	SSlactose (10%)[e]		535.0
	Whey permeate (10%)[e]		711.0
	SSlactose (5%)[e]		849.0

[a] Expressed as nanokatals of hydrolyzed pNPG per gram of substrate
[b] Enzyme production is commercialized
[c] Solid growth medium
[d] Substrate concentration
[e] Lactose concentration

tected in the culture supernatant (350 U ml^{-1}) was significantly higher (around 20 times) than the values obtained with previous strains (Domingues et al. 2000a) in similar conditions (17.5 U ml^{-1}). As a consequence, the metabolism of 10 g lactose l^{-1} occurred in less than 24 h, while for the previously constructed recombinant brewer's strains 150 h were needed. Moreover, the recombinant brewer's strains were not able to metabolize 20 g lactose l^{-1} in 200 h, while with *S. cerevisiae* NCYC869-A3/pVK1.1 the complete metabolism of 50 g lactose l^{-1} was observed in 24 h (see Figs. 2 and 3).

In bioreactor culture, when using an initial lactose concentration of 100 g l^{-1} in the fermentation medium, there was a 300-fold increase in β-galactosidase activity compared with the amount detected using previously constructed strains in shake-flask culture (Domingues et al. 2000a). The high level of β-galactosidase activity detected in the supernatant allows for the rapid metabolizing of lactose, 100 g lactose l^{-1} being consumed in 36 h. Kumar et al. (1992) reported that after 20 h of growth on whey permeate, 26% of the lactose was hydrolyzed by the recombinant strain. With the strain obtained in this work, at the same fermentation time, 57% of the lactose present on cheese-whey permeate was hydrolyzed. The polyploid distiller's yeast transformants constructed by Ramakrishnan and Hartley (1993) grew well in synthetic medium, hydrolyzing 100 g l^{-1} lactose in 50 h. However, a very low fraction of the population retained the plasmid (10%), the β-galactosidase activity detected in the supernatant being very low (comparable with the one obtained with previously reported strains, Domingues et al. 2000a). The flocculent strain *S. cerevisiae* NCYC869-A3/pVK1.1 constructed for *A. niger* β-galactosidase secretion is clearly superior to other, previously constructed strains. It is well-known that the expression level of heterologous proteins is highly dependent on the genetic background of the host strain (Park et al. 2000). In this respect it is worth noting that the low plasmid stability observed in the recombinant polyploid distiller's yeast (Ramakrishnan and Hartley 1993) may be the main reason for the differences observed, as the flocculent recombinant *S. cerevisiae* strain hereby described has greater stability.

When using cheese-whey permeate (initial lactose concentration 50 g l^{-1}) from a Portuguese dairy industry as a substrate, in less than 40 h all the lactose was metabolized, producing 2,635 U extracellular β-galactosidase activity ml^{-1}. Ethanol production was also observed. Doubling the lactose concentration in SSlactose medium resulted in a two-fold higher β-galactosidase activity in the culture supernatant. For this reason, the whey permeate was concentrated two-fold using nanofiltration membranes (150–300 Da cut-off) in order to double the lactose concentration, and assayed as substrate for fermentation and extracellular β-galactosidase production. Lactose was completely metabolized in 80 h compared to 36 h when using semi-synthetic medium as a substrate. The maximum β-galactosidase activity detected in the supernatant was considerably lower (3,214 U ml^{-1}) than that obtained with semi-synthetic medium (5,096 U ml^{-1}). This may be attributed to the presence of a β-galactosidase activity inhibitor in the whey permeate that is active only at high concentrations of the enzyme. For instance, Macris (1981) noted that the concentration of monovalent cations in whey affected β-galactosidase production by *Fusarium moniliforme*. For *A. niger* β-galactosidase, examples of enzyme inhibition by whey have also been reported (Richmond et al. 1981). Trace amounts of divalent cations in the whey were pointed out as a possible cause of this inhibitory effect. Also, Wierzbicki and Kosikowski (1972) observed that heat treatment of whey enhanced lactose hydrolysis. The authors attributed this effect to the heat inactivation of inhibitory substances in the whey or to the formation of new substances that stimulated enzyme activity.

In Table 1 the constructed recombinant strain and other production systems, some implemented commercially, are compared. The maximum enzyme yield using the recombinant strain is the highest, except for the *A. niger* VTT-D-80144 strain. This is a mutant strain se-

Table 2 β-Galactosidase production by the recombinant strain *S. cerevisiae* NCYC869-A3/pVK1.1 and the *A. niger* strains VTT-D-80144 and VTT-D-79106

Strain	β-galactosidase (nkat/ml)	Substrate	Reactor volume (l)	Time (h)	Reference
S. cerevisiae NCYC869-A3/pVK1.1	44	Whey permeate (5%)	2	25	This work
	85	SSlactose (10%)	2	36	
	175	SSlactose (10%, 5 YE[a])	2	60	
A. niger VTT-D-79106	50	Wheat bran (4%)	8	96	Nevalainen (1981)
A. niger VTT-D-80144	165	Wheat bran (4%)	8	96	(Nevalainen (1981)

[a] SSlactose medium with 5 g yeast extract l^{-1}

lected for producing elevated levels of β-galactosidase (Nevalainen 1981). However, if the amount of enzyme activity detected in the supernatant (nkat/ml), instead of enzyme produced per gram of substrate (nkat/g substrate), is considered, the values obtained with the recombinant constructed strain approach those obtained with the *A. niger* mutant strain (Table 2). Moreover, if whey is used as substrate instead of wheat bran, enzyme production by the mutant strain would probably be lower. Even though β-galactosidase is commonly used to cleave lactose into galactose and glucose, its role in nature is more likely in removing β-linked galactose residues from plant-derived oligo- and polysaccharides. de Vries et al. (1999) observed high expression of *lacA* gene on arabinose, xylose, xylan and pectin, while on galactose and galactose-containing oligosacharides (lactose, melibiose, raffinose and stachyose) and polysaccharides (gum arabic, gum karaya, and locust bean gum) expression levels were low. As wheat bran is rich in arabinoxylan and, according to de Vries et al. (1999) *lacA* expression results, the whey substrate for the production of β-galactosidase from *A. niger* probably results in a lower yield than could be obtained using wheat bran as the substrate.

Indeed, by changing the composition of the medium (raising the yeast extract concentration), the β-galactosidase activity detected in the supernatant approaches the activity secreted by *A. niger* (Table 2). Of particular importance is also the fact that the rate of β-galactosidase produced by the recombinant yeast is at least 50% higher than the rate of extracellular β-galactosidase production by moulds. Moreover, *A. niger* is well-known for its ability to produce and secrete a wide range of enzymes – β-galactosidase production being accompanied by other, non-desired proteins. Worthy of consideration is the secretion of proteases by this organism (van den Hondel et al. 1991). Taking all these aspects into consideration, the recombinant constructed strain presents an attractive alternative.

As the enzyme is produced with few protein contaminants (data not shown), a sample of the fermentation broth supernatant (from a fermentation with 10% initial lactose concentration) was concentrated 22 times by Centricon-50 membranes and applied to the cheese-whey permeate (0.5 ml β-galactosidase sample to 10 ml cheese-whey permeate corresponding to a final enzyme concentration of 1.2E4 U ml^{-1}). In 11 h, 74% lactose was hydrolyzed while in 21 h less than 10% of initial lactose remained in solution.

The production system hereby presented seems an interesting one for cheese-whey treatment. While reducing the organic load by hydrolyzing lactose and metabolizing the resulting monosaccharides, the recombinant strain produces β-galactosidase in addition to ethanol. Moreover, as the recombinant strain is flocculent, the use of continuous high-cell-density culture, which has proven its efficiency for ethanol production (Domingues et al. 1999), can be applied for extracellular β-galactosidase production with increased productivity. Further work will focus on continuous high-cell-density operation with this recombinant *S. cerevisiae* strain for heterologous β-galactosidase production.

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